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MEMBRANE PROTEIN MEDIATION OF THROMBOXANE A_2 INDUCED PLATELET AGGREGATION

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Summary: Washed human platelets pretreated with chymotrypsin to remove membrane proteins retain full ability to convert ^{14}C-arachidonic acid to thromboxanes. However, the platelets no longer aggregate when treated with arachidonic acid or calcium ionophore A23187. While thromboxane A_2 may function as a calcium ionophore to mobilize intraplatelet Ca^{2+} and to activate platelets for aggregation, the present studies indicate that thromboxane A_2 induced platelet aggregation is a process mediated by membrane proteins.

Aggregation of human platelets to form a hemostatic plug at the site of vascular injury is essential for primary phase hemostasis (1). Thromboxane A_2 (TXA_2), a prostaglandin endoperoxide derived from arachidonic acid, is a primary intraplatelet mediator for aggregation and the release reaction of platelets (2-4). Platelet aggregating agents such as thrombin, ADP, collagen, and epinephrine all activate TXA_2 synthesis (5-8). Platelet aggregation induced by these agents is also completely or partially abolished by inhibitors of cyclo-oxygenase (9-11) which is essential for synthesis of precursors of TXA_2. However, it is not known how TXA_2 activated platelets interact with each other to form platelet aggregates. We report here that washed human platelets pretreated with various proteases to remove membrane proteins fail to aggregate when exposed to arachidonic acid (0.3 mM) or calcium ionophore A23187 (0.5 μM). TXA_2 synthesis, however, is well preserved in both control platelets and protease pretreated platelets. These results demonstrate that platelet aggregation induced by TXA_2 or calcium ionophore is a process mediated by membrane protein(s).

METHODS. Human platelets for the present experiments were prepared from citrated venous blood of healthy donors who refrained from drug intake for at least two weeks prior to study. The platelets were washed essentially as described by

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Kinlough-Rathbone et al. (12). The platelets obtained from 20 ml of venous blood were washed first with 12 ml of Tyrodes buffer, pH 7.4, containing EDTA (5 mM), apyrase (75 μg/ml) and without Ca^{++} or Mg^{++}. The platelets were then washed again in the same buffer but with the omission of apyrase, and resuspended in 9 ml of standard Tyrodes buffer, pH 7.4. The platelet suspension was divided into three parts and treated with trypsin (100 units/ml) or chymotrypsin (100 units/ml) at 37° C for 30 minutes. Control platelets were incubated with bovine serum albumin (2 mg/ml) under the same conditions. After incubation with protease, four volumes of Tyrodes buffer containing 5 mM EDTA were added and platelets were recovered by centrifugation. The collected platelets were washed once again and finally resuspended in Tyrodes buffer, pH 7.4, containing glucose at 1 mg/ml. The concentration of the platelet suspension was determined by a hemacytometer under a phase contrast microscope.

Synthesis of thromboxane A2 (TXA_2) was studied by utilizing thin layer radiochromatography to monitor the conversion of exogenous [1-^{14}C]-labeled arachidonic acid into TXB_2 which is a stable metabolite of TXA_2. The platelet suspension (10^9 platelets/ml) was incubated with 38.1 nmoles of ^{14}C-labeled arachidonic acid (52.7 mCi/mmole, New England Nuclear Co.) and stirred at 1,000 rpm by a magnetic stirrer at 37°C. After 1 min. the incubation was terminated by adding 50 μl of 1 M citric acid and immediately twice extracted with 2 ml of ethyl acetate. Four ml of ethyl acetate was dried to about 15 μl and reconstituted in 0.2 ml of chloroform/methanol (2:1, v/v). A fraction of this sample was then subjected to thin-layer chromatography on a silica gel G plate (Analtech Inc.) and developed with ethyl acetate/acetic acid (99:1, v/v) twice. The silica gel G plate was scraped every 3 mm from origin by a TLC scraper (Analabs Inc.) and counted in a Nuclear-Chicago scintillation counter. Tritiated or ^{14}C-labeled standards, such as PGE_2, $PGF_{2\alpha}$, TXB_2, PGD_2, PGH_2, HHT, HETE and arachidonic acid, were used to identify the products.

RESULTS AND DISCUSSION. Control platelets (4×10^8 cells) and platelets (4×10^8 cells) pretreated with chymotrypsin or trypsin were solubilized in 4% sodium dodecyl sulfate separately and electrophoresed on a polyacrylamide slab gel (7.5%) using the Laemmli system (13). After electrophoresis, the gel was stained using the PAS technique for glycoprotein (14). As previously reported (15,16), membrane glycoproteins GP I (M_r 145,000), GP II (M_r 120,000), and GP III (M_r 100,000) were reduced significantly for the protease pretreated platelets (data not shown). It is known that thrombin and factor VIII/von Willebrand factor protein induce platelet aggregation through binding to membrane receptors (17,18). In the present study, both thrombin (1 unit/ml) and human factor VIII/von Willebrand factor (2 μg/ml) in the presence of ristocetin (1 mg/ml) failed to aggregate platelets pretreated with trypsin or chymotrypsin. By contrast, both agents aggregated control platelets normally. These results clearly indicate that, in these experiments, platelet membrane proteins were hydrolyzed by the treatment with chymotrypsin or trypsin.

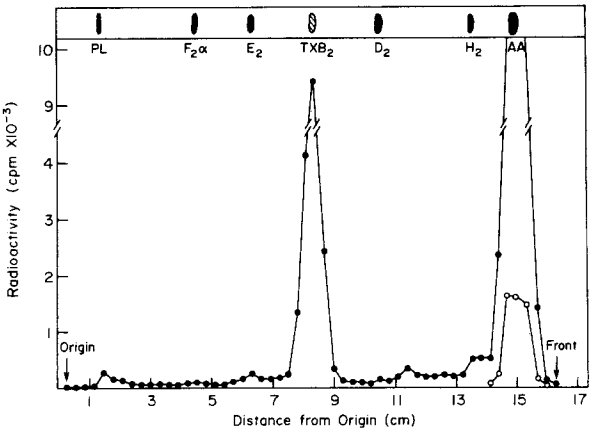

Figure 1. Thin-layer radiochromatogram of products isolated after incubating 38.1 nmole (1-^{14}C) labeled arachidonic acid with 1 ml of platelet suspension (10^6 platelets/µl) at 37° C. Migration of standards is indicated at the top. The following abbrevations are employed: PL, phospholipid; $F_2\alpha$, $PGF_2\alpha$; E_2, PGE_2; TXB_2, thromboxane B_2; D2, PGD_2; H_2, PGH_2; and AA, arachidonic acid. The peak drawn in open circles is 1/10 scale. The percentage of (^{14}C)-arachidonic acid converted to TXB_2 was calculated by dividing radioactivity under the peak of TXB_2 with total radioactivity applied on the silca gel plate. The percentage determined was used to calculate the amount of TXB_2 synthesized. The chromatographic system employed does not resolve HHT and HETE which cochromatograph with arachidonic acid.

Subsequently, TXB_2 synthesis was studied in control, chymotrypsin or trypsin pretreated platelets (Fig. 1). Initially, the rate of synthesis of TXB_2 was determined in each case. Both control and chymotrypsin pretreated platelets had similar time dependent TXB_2 synthesis with a plateau achieved in 45 sec. However, trypsin pretreated platelets did not achieve a plateau until 60 sec. Therefore, the studies reported in Fig. 1 were performed after 60 sec. of incubation of the platelets with [1-^{14}C] - arachidonic acid. These studies demonstrated that TXB_2 synthesis in platelets pretreated with chymotrypsin was identical to that in control platelets (Table 1). However, TXB_2 synthesis in platelets pretreated with trypsin was only 58% of that in control platelets. This result is consistent with previous observations (19,20) that trypsin can act on platelets to release arachidonic acid from the platelet membrane phospholipid, activate the platelet catabolic pathway of arachidonic acid, and consequently inactivate cyclo-oxygenase. In contrast to the finding of Lapetina and Cuatrecasas (20) that TXA_2 synthesis is completely inhibited when horse platelets are exposed

Table I. The Effect of Protease Treatment on Platelet Thromboxane (TX) B_2 Synthesis

	TXB_2 Synthesized	
	EXP. I nmole/ml	EXP II* nmole/ml
Control Platelet	10.7	8.8
Chymotrypsin pretreated platelet	10.3	8.8
Trypsin pretreated platelet	6.1	5.1

*EXP II is the mean of duplicated incubations. The variation between duplicates is less than 5%. The rate of TXB_2 synthesis was also studied for all three kinds of platelets. Maximal TXB_2 synthesis was reached 40 seconds after adding ^{14}C-arachidonic acid into the platelet suspension at 37° C (data not shown). Methods for TXB_2 determination are described in the legend of Fig. 1.

to trypsin, our results demonstrate signifcant preservation of TXA_2 synthesis in human platelets after pretreatment with trypsin. Since no difference in TXA_2 synthesis was detected in control and chymotrypsin pretreated platelets, proteolysis by chymotrypsin is confined to membrane proteins without entry through the plasma membrane to inactivate the TXA_2 synthetic pathways. Furthermore, this result indicates that membrane proteins are not required for arachidonic acid to traverse the platelet plasma membrane.

We then studied arachidonic acid or calcium ionophore A23187 induced aggregation of control and protease pretreated platelets. Aggregation of control platelets was first studied over the range of 0.075 mM to 0.6 mM arachidonic acid or 0.1 μM to 1.0 μM A23187. The optimal concentrations of 0.3 mM arachidonic acid and 0.5 μM A23187 were then used to study the aggregation of protease pretreated platelets. As shown in Fig. 2, control platelets aggregated as expected; however, platelets pretreated with chymotrypsin or trypsin failed to aggregate. Since protease pretreated platelets still synthesize TXA_2, these observations indicate that platelet aggregation induced by TXA_2 is a process mediated by membrane protein(s).

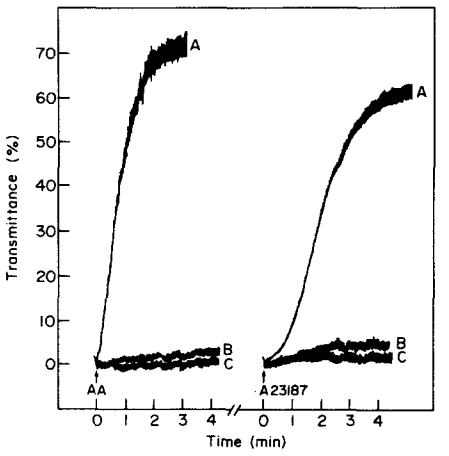

Figure 2. Effect of protease pretreatment on arachidonic acid and calcium ionophore A23187 induced platelet aggregation. Platelet aggregation was studied in a Chrono-Log Model 440 aggregometer at 37° C. The aggregation was initiated by adding 50 µl of arachidonic acid (3.0 mM) or A23187 (5 µM) into 450 µl of a platelet suspension which contained 5 x 10^7 platelets. A, control platelets; B, platelets pretreated with trypsin; C, platelets pretreated with chymotrypsin.

The importance of TXA_2 in inducing platelet aggregation has been well established (2,4,5). However, the mechanism by which TXA_2 activates platelets and induces platelets to aggregate is largely unknown. Striking similarities of morphological changes, release of cytoplasmic granules, and platelet aggregation were reported between platelets stimulated with PGG_2, a precursor of TXA_2, and those treated with calcium ionophore A23187 (21,22). Subsequently, TXA_2 has been shown to enhance Ca^{++} partition from the aqueous phase into organic phase solvents (23,24). The essential role of intraplatelet Ca^{++} mobilization in regulating platelet release reactions and aggregation has also been demonstrated (3,25,26). Hence, it has been postulated that TXA_2 might function as an intracellular Ca^{++} ionophore to mobilize Ca^{++} from its storage sites in the dense tubular system into the cytosol. In view of these findings and our results, it is likely that TXA_2 mobilizes intraplatelet Ca^{++}, and this Ca^{++} mobilization may directly or indirectly activate membrane protein(s) to initiate interplatelet recognition and subsequent aggregation.

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